New tools for diet analysis: nanopore sequencing of metagenomic DNA from rat stomach contents to quantify diet

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Abstract

Background: Using metagenomics to determine animal diet offers a new and promising alternative to current methods. Here we show that rapid and inexpensive diet quantification is possible through metagenomic sequencing with the portable Oxford Nanopore Technologies (ONT) MinION. Using an amplification-free approach, we profiled the stomach contents from wild-caught rats.

Results: We conservatively identified diet items from over 50 taxonomic orders, ranging across nine phyla that include plants, vertebrates, invertebrates, and fungi. This highlights the wide range of taxa that can be identified using this simple approach. We calibrate the accuracy of this method by comparing the characteristics of reads matching the ground-truth host genome (rat) to those matching diet items, and show that at the family-level, false positive taxon assignments are approximately 97.5% accurate. We also suggest a way to mitigate for database biases in metagenomic approaches. Finally, we implement a constrained ordination analysis and show that we can identify the sampling location of an individual rat within tens of kilometres based on diet content alone.

Conclusions: This work establishes proof-of-principle for long-read metagenomic methods in quantitative diet analysis. We show that diet content can be quantified even with limited expertise, using a simple, amplification free workflow and a relatively inexpensive and accessible next generation sequencing method. Continued increases in the accuracy and throughput of ONT sequencing, along with improved genomic databases, suggests that a metagenomic approach to quantification of animal diets will become an important method in the future.

Keywords: Metagenomics, nanopore, diet analysis, rat, next generation sequencing

Background

Accurate quantification of animal diets yields critical insight into ecosystem and food web dynamics. However, unbiased and sensitive assessment of diet content is difficult to achieve. This is largely due to the limited accuracy of many current methods. Such methods include visual inspection of gut contents (1,2), which presents bias against items are most easily degraded (for example, soft-bodied species); stable isotope analysis (3,4), which yields only broad information on diet, such as whether diet items are terrestrial or marine in origin (5,6); and time-lapse video (7,8), for which species identification is difficult for small prey items or in low-light conditions.

To circumvent these issues, DNA-based methods (9,10) have become popular. Perhaps the most widely applied DNA-based method is metabarcoding. This approach relies on PCR amplification and sequencing of conserved regions from nuclear, mitochondrial, or plastid genomes (9). With adequate primer selection, this method can detect a wide range of species, and does not require specific expertise, which is often necessary for other methods.

However, DNA metabarcoding is not free from bias. PCR primers must be specifically tailored to particular sets of taxa or species (11). Although “universal” PCR primer pairs have been developed (for example targeting all bilaterians or even all eukaryotes (12), all primer sets exhibit bias towards certain taxa. Five-fold differences in fungal operational taxonomic units (OTU) estimates have been found when using different sets of fungal-specific PCR primer pairs (13). It has also been shown that published

universal primer pairs were capable of amplifying only between 57% and 91% of tested metazoan species, with as few as 33% of species in some phyla being amplified at all (e.g. cnidarians)(14). Different genomic loci from the same species can exhibit up to 2,000-fold differences in DNA concentration as inferred using qPCR (15). The choice of polymerase can also bias diversity metrics when using metabarcoding (16). For these reasons, an approach that circumvents PCR and thus avoids these biases is desirable.

Metagenomic sequencing aims to directly sequence all of the DNA in a sample without introducing bias. Although there are still biases with this approach, for example due to nucleotide content affecting the likelihood of a molecule being sequenced, these are inherently less than those introduced by metabarcoding. Metagenomic approaches have most frequently been used to yield insights into microbial diversity and function (17–24). Metagenomic applications aimed at eukaryotic taxa identification are less common. Several metagenomic diet studies have implemented filtering steps to select only mitogenomic sequence or metabarcode regions, or have used abridged databases before data analysis in order to mitigate database biases (25–27). However, to our knowledge, very few studies have used unfiltered metagenomic sequence analysis to infer diet (30,31).

Here, we establish a proof-of-principle methodology to accurately classify metagenomic sequences from eukaryotic taxa and determine diet content using low-accuracy, long-read sequencing, Oxford Nanopore (ONT).

We quantified rat diets from several locations in the North Island of New Zealand using stomach samples. Using these samples and methodology provides three distinct advantages.

First, rats are extremely omnivorous. As such, they serve as an excellent means to quantify the breadth of taxa that can be detected using a metagenomic long read approach.

Second, the use of stomach samples means that a significant number of reads will be host reads. This allows us to assess the characteristics of true positive sequence reads (rat-derived reads that match rat database sequences), as well as false positive reads (rat-derived reads that match non-rat database sequences). We can then determine whether reads matching diet items have similar characteristics to known true positive reads. This use of host reads is exactly analogous to feeding the rats a diet of known content (i.e. rat) and testing whether the contents of the known diet can be accurately identified using this method.

Third, quantifying rat diets has important ecological implications. It is well-established that the relatively recent introduction of mammalian predators to New Zealand has had significant negative effects on many of the native animal populations. This ranges from insects (34), to reptiles (35), to molluscs (36), to birds (37,38), with downstream effects on terrestrial and aquatic ecosystems (39). To counteract the effects of mammalian predators, an ambitious plan is currently being put into place that aims for the eradication of all mammalian predators from New Zealand (including possums, rats, stoats, and hedgehogs), by 2050 (http://www.doc.govt.nz/predator-free-2050; (40). A

useful step toward this goal would be to prioritise the management of predators and establish in which locations native species experience the highest levels of predation. To do so requires establishing the diet content of local mammalian predators.

Importantly, using DNA sequencing methods to profile diet does not require high depth to accurately profile the breadth of diet items consumed by an animal. Here we aim to quantify all diet items present in the diet at 1% or more. At this fraction, if we assume that read counts are Poisson distributed, with only 2,000 reads, 99% of the time we will quantify such items within 2-fold of their true amount. Thus, diet quantification presents a clear example of a situation in which sequencing depth is not a critical factor.

Results

DNA sequencing and assignment of reads to taxa

We selected eight rats from each of three locations near Auckland, New Zealand for diet quantification. Each location comprised a different type of habitat: undisturbed inland native forest (Waitakere Regional Parklands, WP); native bush surrounding an estuary (Okura Bush Walkway, OB); and restored coastal wetland (Long Bay Regional Park, LB). We isolated DNA from whole stomach contents from each rat (see Methods). We obtained DNA from entire homogenised stomach samples. We sequenced these DNA samples using the Oxford Nanopore ligation sequencing kit (LSK-108) and native barcoding kit to multiplex the samples within a sequencing run. We performed two separate runs, obtaining a total of 82,977 reads (January 2017) and 96,150 reads (March 2017) from each. These numbers are not far below expectations given the flow cell and kit chemistry and MinKNOW software versions available at that time (47).

122 However, these read numbers are considerably below those expected for current ONT
123 flow cells and software, which has improved per flow cell output more than 100-fold
124 above these numbers.

125 We found large variation in the numbers of reads per barcode: approximately 10-fold for
126 the January samples, and up to 40-fold in March (**Fig. 1A** and **1B**). We hypothesise that
127 this is due to the highly variable quality of DNA in each sample. This did not appear to
128 have strong effects on read accuracy, as the median phred quality scores per read
129 ranged from 7-12 (0.80 - 0.94 accuracy) for both runs.

130 The degradation of the DNA during digestion in the stomach, as well as fragmentation
131 during DNA isolation (48) and sequencing library preparation led to relatively short
132 median read lengths of 606 bp and 527 bp for the January and March datasets,
133 respectively (**Fig. 1C**). Within runs, read length and quality were similar. Although these
134 median read lengths are considerably shorter than other nanopore sequencing results
135 from both our and others work (49), we found wide variation in length, with almost 10%
136 of all reads being longer than 1200 bp. Notably, reads of this length can allow more
137 precise taxonomic identification than accurate short reads (32).

138 To quantify diet contents we first BLASTed all sequences against a combined database
139 of the NCBI nt database (the partially non-redundant nucleotide sequences from all
140 traditional divisions of GenBank excluding genome survey sequence, EST, high-
141 throughput genome, and whole genome shotgun
142 (ftp://ftp.ncbi.nlm.nih.gov/blast/db/README)) and the NCBI other_genomic database
143 (RefSeq chromosome records for non-human organisms
144 (ftp://ftp.ncbi.nlm.nih.gov/blast/db/README)). We used BLAST as it is generally viewed

145 as the gold standard method in metagenomic analyses (50). Of the 133,022 barcoded

146 reads, 30,535 (23%) hit a sequence in the combined nt and other_genomic database at

147 an e-value cut-off of 1e-2.

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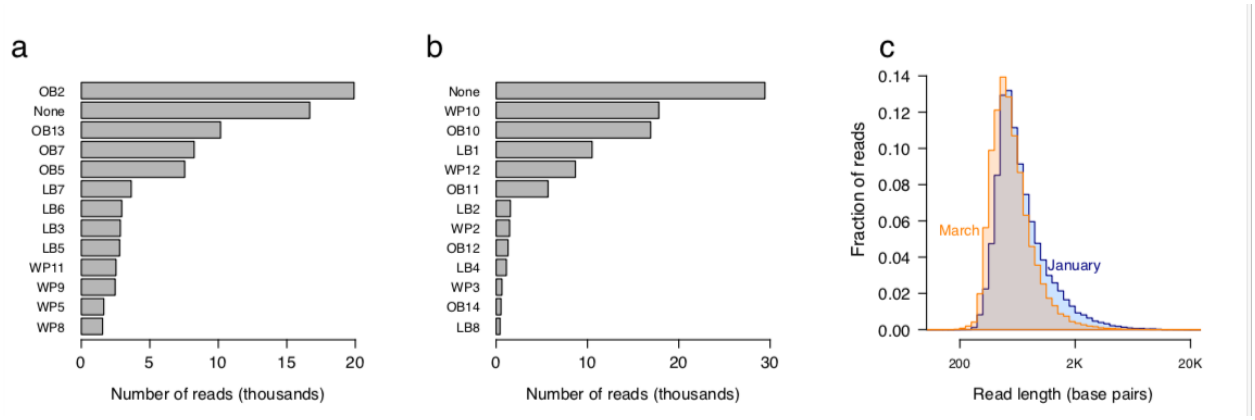

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150 **Fig. 1. Results of nanopore metagenomic sequencing of rat stomach contents. (A)**
151 **and (B) Barcode distributions for January and March runs, respectively.** We
152 multiplexed the samples on the flow cells, using 12 barcodes per flow cell. The
153 distribution of read numbers across barcodes varied by up to 40-fold. 20% (January)
154 and 30% (March) of all reads could not be assigned to a barcode ("None"). The inability
155 to assign these reads to a barcode is due primarily to their lower quality. **(C) Read**
156 **length distribution for January and March nanopore runs.** 90% of the reads were
157 between 350 bp and 1,580 bp in length, with only 0.55% being longer than 4,000 bp.

158 We first aimed to assess the quality of these hits. We found a bimodal distribution of

159 alignment lengths and e-values (**Fig. 2A**). We also noticed that mean read quality had

160 substantial effects on the likelihood of a read yielding a BLAST hit, with almost 40% of

161 high accuracy reads having hits in the March dataset, as compared to 1% of low

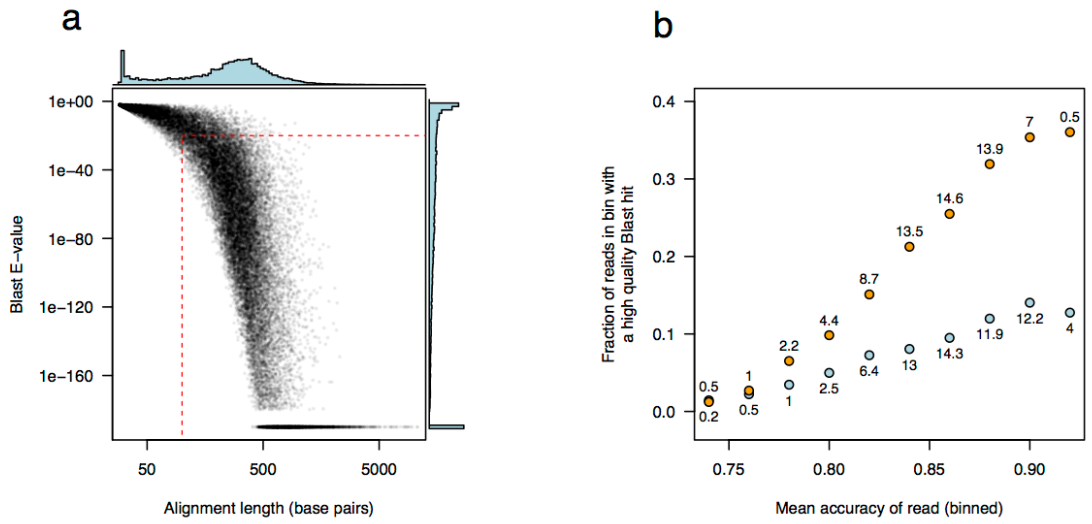
162 accuracy reads (**Fig. 2B**). We hypothesized that many of the short alignments with high

163 e-values were false positives. We thus first filtered this hit set, only retaining BLAST hits

164 with e-values less than 1e-20 and alignments greater than 100 bp. To give some

165 intuition for this length cut-off, a 100bp read with five 1 bp indels and seven mismatches

166 (88% identity) would have an identical raw score and e-value (given the default match,
167 mismatch, and gap parameters) to a single-end 70 bp Illumina read with a 100%
168 identical database match. Similar or quality filters based on length and identity have
169 been imposed previously (30). A total of 22,154 hits passed this e-value filter.



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171 **Fig. 2. BLAST hits of metagenomic reads. (a)** Alignment lengths and e-values were
172 bimodally distributed. The y-axis is plotted on a log scale, with zero e-values
173 suppressed by adding a small number (1e-190) to each e-value. The horizontal red
174 dotted line indicates the e-value cut-off we implemented and the vertical red dotted line
175 indicates the length cut-off (e-value < 1e-20 and alignment length of 100, respectively)
176 to decrease false positive hits. **(b) The fraction of reads with high quality BLAST hits**
177 **(e-value < 1e-20) increased as a function of read accuracy.** We binned the data
178 according to mean read accuracy (bin width = 0.02) and calculated the fraction of reads
179 within each bin that have a high quality BLAST hit (alignment length greater than 100bp
180 and e-value less than 1e-20) for the January and March runs separately (blue and
181 orange points, respectively). The number of reads in each bin is indicated above each
182 point (in thousands). There is a clear positive correlation between mean accuracy and
183 the likelihood of a high quality BLAST hit, reaching almost 40% for high accuracy reads
184 (>92.5%) for the March dataset.

185

186 We next used MEGAN6 (41) to assign reads to specific taxa. MEGAN6 employs an
187 LCA algorithm to assign reads to a taxon. For example, if a read has BLAST hits to five

188 different species, three of which have bit scores within 20% of the best hit, the read will
189 be assigned to the genus, family, order, or higher taxon level that is the LCA of those
190 best-hit three species (51). If a read matches one species far better than any other, by
191 definition, the LCA is that species.

192 16,820 reads (76%) were assigned to a taxon by MEGAN. Of these, 31% were
193 assigned by MEGAN as being bacterial, and 55% of these were *Lactobacillus spp*.
194 These results match previous studies on rat stomach microbiomes, which have found
195 lactobacilli to be the dominant taxa (52–55). Plant-associated *Pseudomonas* and
196 *Lactococcus* taxa were also common, at 7% and 6%, respectively.

197 MEGAN assigned reads to a wide range of eukaryotic taxa. To conservatively infer
198 taxon presence, we first reclassified MEGAN species-level assignments to the level of
199 genus. Even so, many clear false positive assignments remained (e.g. hippo and naked
200 mole rat). These matches were generally short and of low identity. To reduce such false
201 positive taxon inferences, we used information from reads assigned to the genera
202 *Rattus* (rat) and *Mus* (mouse).

203 We inferred that the reads assigned to *Rattus* (2,696 reads in total) were true positive
204 genus-level assignments and that the reads assigned to *Mus* (2,798 reads in total) were
205 false positive genus-level assignments (and not true positive *Mus*-derived reads). If
206 these were true positive *Mus* reads, then they would be due to mouse predation by
207 some of the rats. Although rats are known to prey on mice (56), if this had occurred, we
208 would expect that (1) if a rat predated a mouse the ratio of mouse to rat reads would be
209 higher than in rats that had not predated mice; (2) in those same rats, the percent

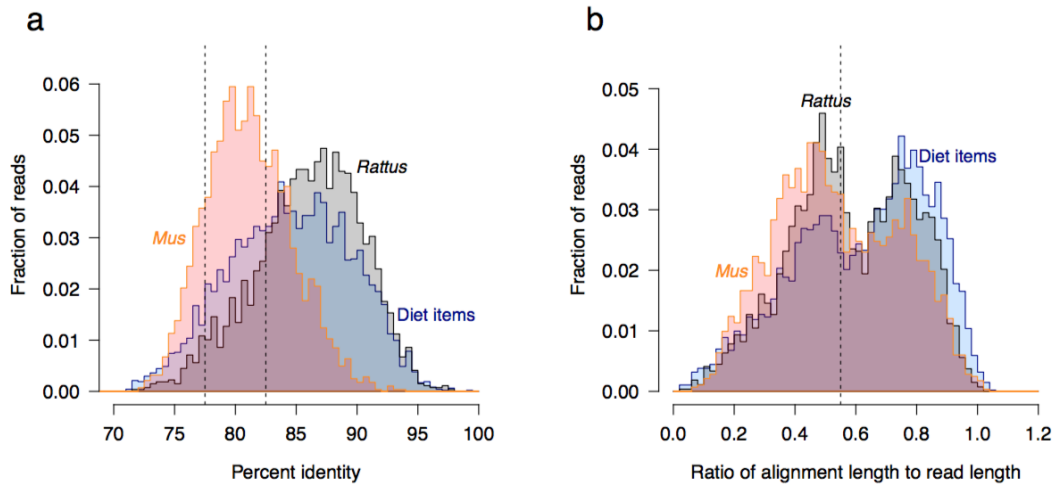
210 identity of the reads assigned to *Mus* would be higher than in rats that had not predated
211 mice. However, we found that the ratio of mouse to rat reads and percent identity was
212 similar for all rats. This suggested that these *Mus* hits were indeed false positives and
213 could thus be used to determine the rate of false positive genus-level assignment.

214 The mean percent identity values of the best BLAST hits for *Rattus* and *Mus* reads
215 differed substantially, with *Rattus* reads having a median identity of 86.4%, and *Mus*
216 81.0% (**Fig. 3A**). The mean percent identity for *Rattus* reads corresponds very well to
217 that expected given the mean quality scores of the reads (86.4% identity corresponds
218 to a mean quality score of 8.7, similar to what we observed; **Fig. S1A-C**).

219 To further investigate alignment characteristics differentiating true positive (*Rattus*) and
220 false positive (*Mus*) assignments, we considered the ratio of alignment length to read
221 length. If the full read aligns, this ratio is one. More generally, higher quality alignments
222 should have higher ratios. Indeed, we found this ratio differed substantially between
223 the *Rattus*- and *Mus*-assigned reads: the median ratio of alignment length to read
224 length was 0.57 for *Rattus* and 0.52 for *Mus* (**Fig. 3B**).

225 We used this analysis to select cut-off values for assigning reads as having true
226 positive or false positive taxon assignments. For genus-level assignments, we required
227 at least 82.5% alignment identity, and an alignment length to read length ratio of at
228 least 0.55. These cut-offs exclude 88% of the reads falsely assigned to *Mus*, instead
229 correctly assigning them one taxon level higher, to the Family *Muridae*.

230 However, this rate of false positive assignments is still relatively high. We thus also
231 quantified the rate of false positive assignment at the level of family. We first identified
232 all reads classified as being from the order *Rodentia.* We then implemented specific
233 cut-offs, requiring reads and database matches to have 77.5% identity, an alignment
234 length to read length ratio of at least 0.1, and a total alignment length of at least 150
235 bp. With these cut-offs, 97.3% of all reads assigned to the Order *Rodentia* were
236 classified in the likely true positive Family *Muridae* (which contains the genus *Rattus*).
237 The remaining 2.7% were assigned to the family *Cricetidae* (voles and lemmings),
238 except or four reads assigned to *Spalacidae* (mole-rats) (**Fig. S2**). These are clear false
239 positive assignments, as it is highly unlikely that these families were predated.
240 However, these results establish that by implementing specific cut-off values for



241

242 **Fig. 3. Distributions of percent identity and length for alignments of reads**
243 **matching *Rattus* (rat), *Mus* (mouse), and diet items. (a) The percent identity for**
244 **alignments of rat (*Rattus*) and diet items is much higher than for mouse (*Mus*).**
245 Histograms of the percent identity of the alignment of the top BLAST hit with the read.
246 *Mus* matches have substantially lower percent identity compared to both *Rattus* and diet
247 items. The dotted lines indicate the cut-offs that we implemented for inferring reads as
248 belonging to a specific genus (above 82.5% identity) or family (above 77.5% identity).
249 **(b) Ratios of alignment lengths to read lengths of rat (*Rattus*) and diet items are**

higher than for mouse (*Mus*). This plot is analogous to that in (a). The dotted line indicates the cut-off that we implemented for inferring reads as belonging to a specific genus (above 0.55).

alignments, we can ensure a low rate of false positive assignments at the family level, especially for taxa with genomic sequence well-represented in the database.

However, it is possible that for taxa that are not well-represented in databases, read classification will be less accurate. If this occurred, we would expect that reads would frequently be assigned to the wrong taxon, resulting in lower percent identities and low alignment lengths for those reads. We thus checked whether diet items had percent identities and alignment lengths similar to the true positive *Rattus* alignments, or instead whether they were more similar to the false positive *Mus* alignments. We found that the majority of diet items had alignment percent identities that overlapped with the *Rattus* reads. Furthermore, the alignment length to read length ratios often exceed those for the *Rattus* reads. This suggests that the diet taxa assignments are often correct down to the level of genus (as the *Rattus*-assigned reads are correct to the level of genus) and are not false positive assignments. However, here we conservatively report diet items at the level of family.

For reads that did not pass the above cut-offs, we placed taxon assignments at the level of order, or used the taxon level assigned by MEGAN. Using these cut-offs, 16% of all reads were classified at the genus level; 71% were classified at the family-level or below; 89% were classified at the order-level or below; and 98% were classified at the phylum-level or below.

272 Beyond the small fraction of false positive family assignments within *Rodentia*
273 (discussed above), a small fraction of clear false positives remained in other orders.
274 Again, most had short alignments (**Table S4**). The exception to this were three reads
275 from two rats matching *Buthidae* (scorpions), which had alignment lengths of 762 bp,
276 664 bp, and 298 bp with identities of 83%, 88%, and 79%, respectively. It is unlikely
277 these are true positives, and instead we hypothesise that these rats predated
278 harvestmen (*Opiliones*), a closely related sister taxon within *Arachnida*, but lacking
279 significant amounts of genomic data. Despite the presence of these false positive taxa,
280 we did not further increase the stringency of our filters, suggesting that we have a small
281 rate of false positive inference at the family level (2.7% in the case of *Muridae*), and
282 most likely similar rates for other family assignments.

283 Identification of diet

284 Within each rat, we identified a wide variety of plant, animal, and fungal orders, ranging
285 from two to 25 orders per rat (mean 8.7; **Fig. 4**). In total, we identified taxa from 68
286 different Families, 55 different Orders, 15 different Classes, and eight different Phyla
287 (**Fig. 5**). In this analysis, we consider all non-host, non-bacterial reads as potential diet
288 items, although not all are necessarily so. Plants were the primary diet item, with the
289 largest fraction of rats consuming four predominant orders: *Poales* (grasses), *Fabales*
290 (legumes), *Arecales* (palms), and *Araucariales* (podocarps). The dominance of plant
291 matter (fruits and seeds) in rat diets has been established previously (57,58). Animal
292 taxa made up a smaller component of each rat's diet, with *Insecta* dominating:
293 *Hymenoptera* (bees, wasps, and ants), *Coleoptera* (beetles), *Lepidoptera* (moths and
294 butterflies), *Blattodea* (cockroaches), *Diptera* (flies), and *Phasmatodea* (stick insects). In

295 addition, *Stylommatophora* (slugs and snails) were present in substantial numbers (**Fig.**
296 **5A** and **5B**). Fungi were only a small component of the rats' diet, although several
297 orders were present: *Sclerotiniales* (plant pathogens), *Saccharomycetales* (budding
298 yeasts), *Mucorales* (pin molds), *Russulales* (brittlegills and milk-caps), and
299 *Chytotheriales* (black yeasts). Finally, for many rats, a substantial proportion of the
300 stomach contents were parasitic worms (primarily *Spirurida* (nematodes) and
301 *Hymenolepididae* (tapeworms)).

302 Due to our metagenomic approach, the fraction of each element of the rats' diets is
303 distorted by biases in genomic databases: whole genome data exists for only a few
304 taxa, while mtDNA, rDNA, metabarcode loci, and microsatellite sequence data are
305 present in the database for many animal and plant genera. To quantify database-driven
306 bias, for each taxon we determined the fraction of hits that mapped to mtDNA, rDNA,
307 microsatellites, or EST libraries (we refer to this as *non-genomic*), versus the fraction of
308 hits that mapped to DNA sequences annotated as arising from genome sequencing
309 projects). We expect that for animals with sequenced genomes, these two fractions
310 should be approximately constant, largely determined by the relative amounts of mtDNA
311 and nuclear DNA in the cell. For animals without sequenced genomes, there should be
312 considerably more hits to mtDNA, plastid, rDNA, and microsatellites (non-genomic
313 sequence), and few (if any) genomic hits. By comparing these fractions for taxa with
314 complete genome sequences in the database to taxa without complete genomes we
315 aimed to assess the effects of this bias.

316

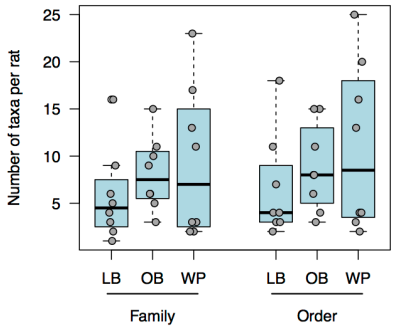
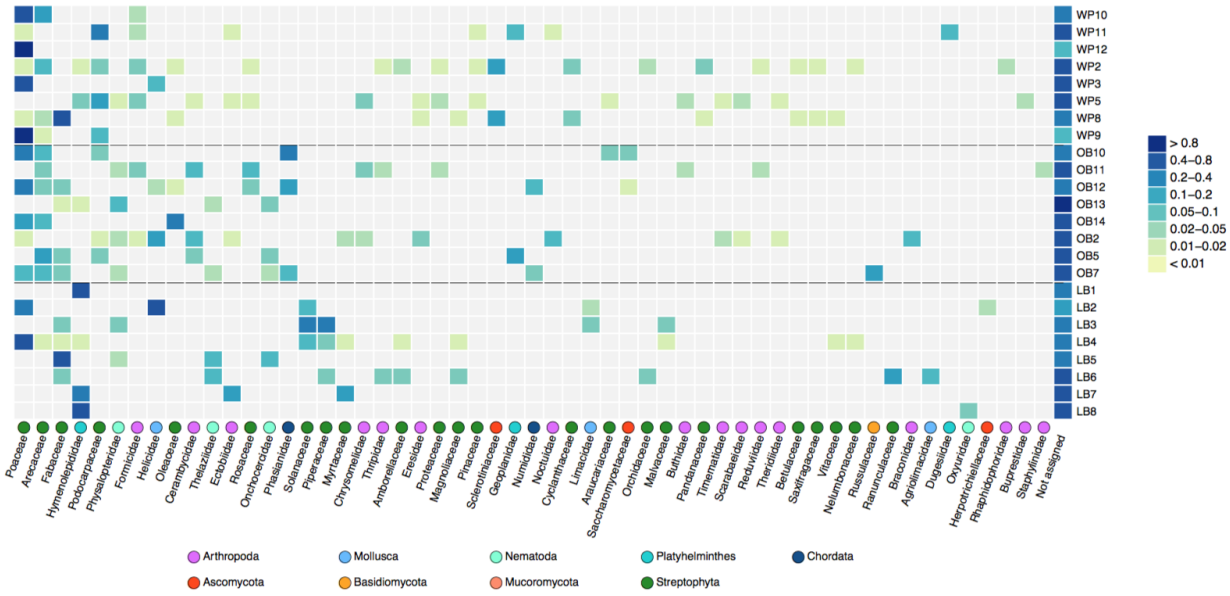


Fig. 4. Numbers of taxa in individual rats. Each boxplot indicates the range of families (left boxes) or orders (right boxes) consumed by each rat in each location (OB: Okura Bush, native bush; LB: Long Bay Park, restored wetland; WP: Waitakere Park, native forest). The numbers for individual rats (eight per location) are plotted in grey.



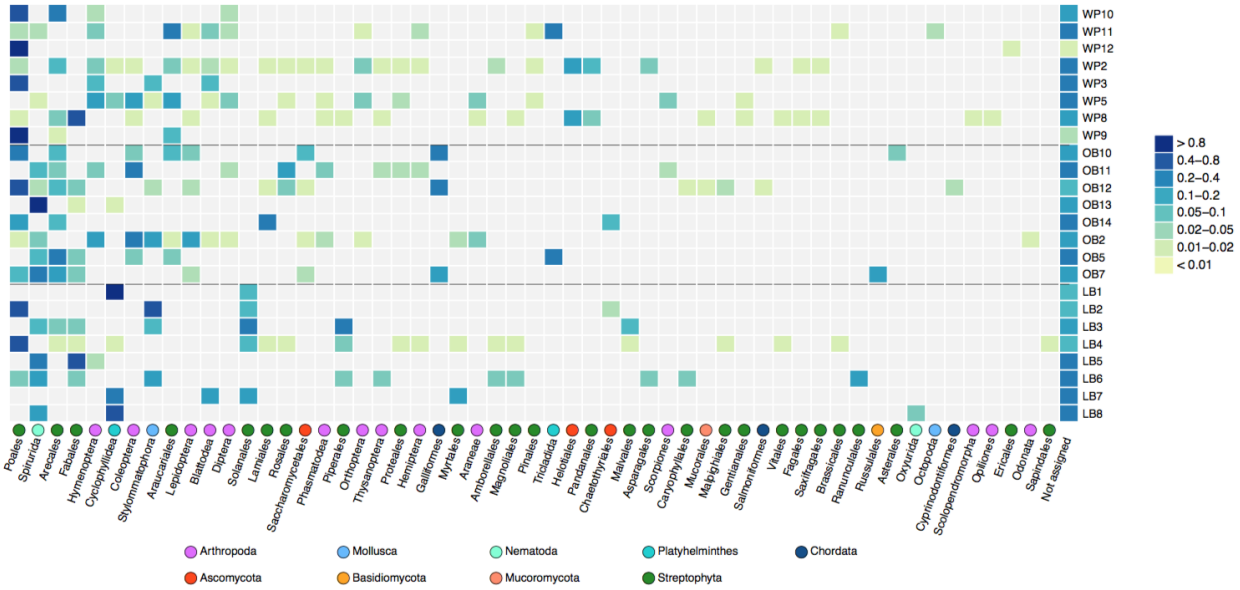


Fig. 5. Proportions of taxa in the diets of individual rats. (a) Reads assigned to taxa at the family and (b) order level. The rows correspond to a single rat, with the proportions of reads for that rat assigned to each family or order indicated in shades of blue and yellow. Reads that were not assigned to a specific family or order are indicated at the right side of the figure. The families and orders have been sorted so that the most common diet components appear on the left. Only the 55 most common families are shown. Note that the color gradations presented on the scale are not linear.

We first considered only animals with sequenced genomes in the database. For these, we found that the fraction of reads that mapped to non-genomic sequence (mtDNA, rDNA, microsatellite, plastid, and EST library) ranged from 0% (*Coturnix* and *Numida*) to 27% (*Rattus*) to 39% (*Gallus*) (**Fig. 6**). This is a considerable range, and we hypothesise that variation in the fraction of non-genomic reads is due to the type of tissue sequenced. For *Rattus* the sequenced tissue was primarily stomach muscle, which has a relatively high fraction of mtDNA, explaining the large fraction of reads mapping to mtDNA.

For plants with sequenced genomes, the fraction of reads matching non-genomic sequence (mostly mtDNA, plastid, and rDNA) was generally lower: between 2% (*Cenchrus*) and 12% (*Zea*). Thus, on average, for animals with sequenced genomes present in the database, approximately 30% of all reads mapped to non-genomic sequences; for plants, approximately 5% mapped to non-genomic sequences.

In contrast, for taxa with little or no genomic sequence in the database, the vast majority of matches were non-genomic (mtDNA, plastid, rDNA, or microsatellite loci): 90% of *Phoenix* (date palm) hits; all *Helix* (snail); and all *Rhaphidophorae* (cave weta) hits. All *Arthurdendyus* (the endemic New Zealand flatworm) hits were solely to rDNA loci.

This overrepresentation of non-genomic sequence is in strong contrast to animals that do have sequenced genomes. In that case we expect only 30% of all reads should map to non-genomic sequence. Thus, these results indicate that for animal taxa with little or no genomic sequence data, we have underestimated the actual number of sequences from that taxon by approximately two- to three-fold. For plant taxa with little or no genomic sequence data, we have likely underestimated read abundance by approximately 20-fold.

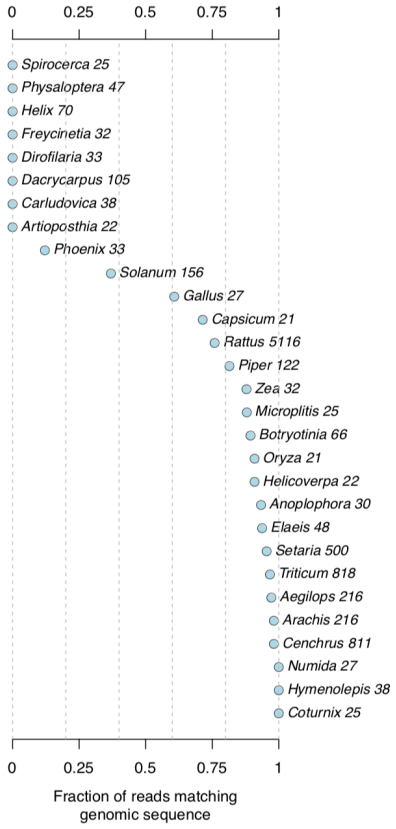


Figure 6. Fractions of reads matching genomic and non-genomic sequence for the best BLAST hit of each read. For the species with complete genomes, the fraction of reads matching genomic sequence ranges from 40% (*Solanum*) to 100%. This large range is likely due to the tissue from which the DNA was isolated. For example, muscle tissue has a higher fraction of mtDNA to nuclear DNA than egg. For species without fully sequenced genomes, this fraction ranges from 0% to 20% (*Phoenix*, which has a small amount of genomic data present in the database).

After classification of the reads at the genus, family, and order level, further examination of the data suggested that specific taxa were overrepresented in the diets of rats from particular locations. For example, six out of eight rats from the native estuarine bush habitat (OB) consumed *Arecaceae*, while only one in the restored wetland area (LB) did. All three rats that consumed *Phaseanidae* were from the native estuarine habitat (OB). All five rats that consumed *Solanales* were from the restored wetland area. These

372 patterns suggested that it might be possible to use diet components alone to pinpoint
373 the habitat from which each rat was sampled.

374 nMDS and CAP analysis by location

375 In order to determine if diet composition of the rats differed consistently between
376 locations, we first performed an unconstrained analysis using non-metric
377 multidimensional scaling (nMDS) on taxa assigned at the family level. The input for the
378 nMDS was the dissimilarity matrix (Bray-Curtis distance of diet at the family level).
379 NMDS uses rank-based distances to cluster samples that are most similar.

380 The family-level unconstrained ordination (nMDS) showed no obvious grouping of rats
381 with respect to the locations (**Fig. 7A**), indicating that locations did not correspond to the
382 predominant axes of variation among the diets. We next performed a constrained
383 ordination method, Canonical Analysis of Principal coordinates (CAP, see Methods).
384 CAP identified axes of variation, if any, that distinguish the diets of rats from different
385 locations (**Fig. 7B**). We found that the CAP axes correctly classified the locations of 19
386 out of 24 (79%) rats using a leave-one-out procedure. The families having the largest
387 correlations with the first two principal coordinates, and thus most responsible for the
388 separation between groups, were primarily plants: *Arecaceae*, *Podocarpaceae*,
389 *Piperaceae*, and *Pinaceae*. In addition, insect groups (*Cerambycids* and *Formicids*) and
390 birds (*Phaseanidae* and *Numididae*) played a role (**Fig. 7C**).

391 The families driving similarity within the three locations (i.e., those that had the greatest
392 within-location SIMPER scores, see Methods) varied among locations. LB had average
393 Bray-Curtis within-location similarity of 13%; mostly attributable to *Hymenolepidae*

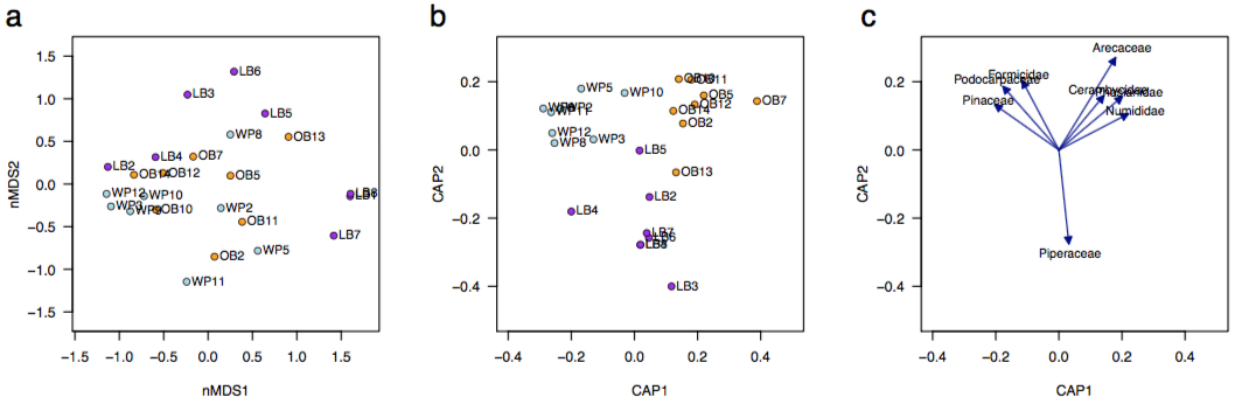
394 (accounting for 51% of the within-group similarity), *Solanaceae* (11%), and *Fabaceae*

395 (11%). The average similarity for OB was 21%, with the greatest contributing taxa

396 being *Arecaceae* (33%), *Poaceae* (23%), *Fabaceae* (9%), and *Phasianidae* (8%). The

397 average similarity for WP was 24%, with the greatest contributing taxa being *Poaceae*

398 (72%) (**Table S4**).



399

400 **Fig. 7. Unconstrained nMDS (a) and constrained CAP (b) ordinations of the diets**
401 **of rats from three locations. Both ordinations were based on Bray-Curtis**
402 **dissimilarities of square root transformed proportions of reads attributed to each**
403 **family.** The locations were a native estuarine bush (OB, orange); a restored marine
404 wetland (LB, purple); and a native forest (WP, light blue). The CAP ordination is
405 repeated in panel **(c)** as a biplot with the rats omitted to show the Pearson correlations
406 between families and the first two CAP axes. The eight families with the strongest
407 correlations are shown, indicating the taxa associated with each location.

408

409 ## Discussion

410 ### Accuracy and sensitivity

411 Here we have shown that using a simple metagenomic approach with error-prone long

412 reads allows rapid and accurate classification of rat diet components (approximately

413 2.7% error in taxon assignments at the family-level). We expect that this technique can

414 be used to infer diet for a wide variety of animal and sample types, including samples

415 that use less invasive collection methods, such as fecal matter. The sensitivity of this
416 approach will likely improve as the accuracy and yield of ONT sequencing continues to
417 increase. The analysis here is based on less than 200,000 reads from two flow cells.
418 Current yields for similar read length distributions are in excess of ten million reads per
419 flow cell. As ONT sequencing accuracy improves (currently just above 96% modal
420 accuracy), this will also increase the applicability of this method. This is illustrated by
421 the fact that the fraction of reads yielding BLAST hits increased substantially for higher
422 quality reads, approaching 40% for high quality reads in our dataset (reads with greater
423 than 92.5% accuracy, **Fig. 2B)**.

424 As the species sampling of genomic databases increases (59), the taxon-level
425 precision of this method will improve. Given the current rate of genomic sequencing,
426 with careful sampling, the vast majority of multicellular plant and animal families (and
427 even genera) will likely have at least one type species with a sequenced genome within
428 the next decade. Continued advancement in sequence database search algorithms as
429 compared to current methods (23,24,60) should considerably decrease the
430 computational workload necessary to find matching sequences.

431 Methodological advantages

432 As genomic databases become more complete, metagenomic approaches will offer
433 significant advantages due to decreased bias. We found that rats consumed many soft-
434 bodied species (e.g. mushrooms, flat worms, slugs, and lepidopterans) that would be
435 difficult to identify using visual inspection of stomach contents. Achieving data on such
436 a wide variety of taxa (across multiple phyla) would be difficult to quantify using

437 metabarcoding, as there are no universal 18S or COI universal primers capable of
438 amplifying sequences in all these taxa. While it might be possible to use several
439 different primer sets targeted at different phyla or orders, quantitatively comparing diet
440 components across these using sequences amplified with different primer sets is
441 extremely difficult due to differences in primer binding and PCR efficiency. A second
442 advantage of metagenomic sequencing (as has been pointed out previously), is that it
443 allows for “holistic” views of ecology, as it simultaneously profiles parasites,
444 microbiome, and prey species (30).

445 The ONT-based sequencing method has several unique advantages, and perhaps the
446 most obvious is the accessibility of the platform. Compared to other high throughput
447 sequencing technologies (e.g. Illumina, IonTorrent, or PacBio), there is no initial capital
448 investment required to use the platform. On a per-sample basis, data generation is
449 inexpensive (assuming 12 multiplexed samples, approximately $150 USD per sample,
450 and half this price if reagents are purchased in bulk). Library preparation and
451 sequencing can be extremely rapid, going from DNA sample to sequence in less than
452 two hours (61). Furthermore, the sequencing platform itself is highly portable. Given (1)
453 that ONT-based methods are now similar in cost-per-read (approximately $650 for 10
454 million reads) than the most accessible Illumina method (MiSeq, approximately $1300
455 for 20 million reads); and (2) that even marginal increases in read length are likely to
456 significantly improve species identification, we expect that ONT-based methods should
457 soon become useful for routine ecological monitoring of species (62).

458 Some modifications to our approach might further increase the precision of our ability to
459 infer community composition. Any error-prone long read dataset (i.e. PacBio or ONT)
460 has both short (e.g. 500 bp) and long (e.g. 5000 bp) reads, as well as high quality (e.g.
461 mean accuracy greater than 90%) and low quality (e.g. mean accuracy less than 80%)
462 reads. When inferring community composition, a null expectation is that taxa should be
463 equally represented by long, high quality reads as they are by short, low quality reads. If
464 some taxa are represented only by short, low quality reads, this suggests that these
465 taxa may be false positive inferences. Similarly, the difficulty in correctly mapping short
466 inaccurate reads could be mitigated by weighting the probability of taxon mapping by
467 the number of long, accurate reads that map to certain taxa. Thus, the fact that not all
468 reads are extremely long and accurate does not mean that they cannot all be used to
469 infer taxon presence in metagenomic analyses.

470 For many diet studies, the aim is to ideally resolve the biomass, or nutritional content, or
471 prey numbers. However, as a caveat to the methods utilised here, and to all
472 sequencing-based methods, the accuracy of estimating these numbers is constrained
473 by the fact that different tissues and different taxa have different amounts of DNA (both
474 nuclear and mitochondrial) per gram of biomass. It is nearly to impossible to fully
475 account for this variation. Regardless, there is considerable utility in using sequencing
476 based approaches for diet assessment, not least because it is one of the few methods
477 that allows the full breadth of the diet to be observed, as illustrated here by the number
478 of different orders we find that rats predate.

Conclusions

Here we have shown that a rapid error-prone long read metagenomic approach is able to accurately characterise diet taxa at the family level and distinguish between the diets of rats according to the locations from which they were sourced. This information may be used to guide conservation efforts toward specific areas and habitats in which native species are most at risk from this highly destructive introduced predator.

Methods

Study Areas

We trapped rats from three locations near Auckland, New Zealand. Each location comprised a different type of habitat: undisturbed inland native forest (Waitakere Regional Parklands, WP); native bush surrounding an estuary (Okura Bush Walkway, OB); and restored coastal wetland (Long Bay Regional Park, LB) (**Fig. 8**). Snap traps in OB and LB were baited with peanut butter, apple, and cinnamon wax pellets; or bacon fat and flax pellets.

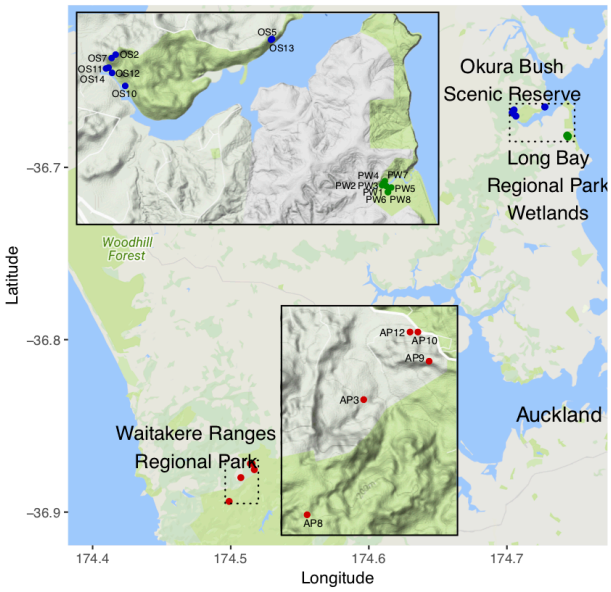


Fig. 8. Location of rat sampling sites in the greater Auckland area in the North

Island of New Zealand. Each point indicates a trap where one rat was captured, with the colour of the points indicating the three broad locations: the native estuarine bush habitat of Okura Bush (OB), the restored wetland of Long Bay (LB), and the native forest of Waitakere Park (WP). The two insets show the three locations in higher resolution with topographical details. Green indicates park areas. Precise geographical coordinates were only available for five out of eight rats in WP.

Traps in WP were baited with chicken eggs, rabbit meat, or cinnamon scented poison pellets. From 16 November to 16 December 2016, traps were surveyed by established conservation groups at each site every 48 hours. A total of 36 rats were collected from these locations. Three of the rats from WP had poison in their stomachs. However, all three of these were killed by the snap traps, and as such are unlikely to have swallowed any bait. In addition, none were identified as having chicken or rabbit in their diet. The majority of rats collected (34/36) were determined to be male *Rattus rattus* by visual inspection. These 34 rats were selected for further analysis.

DNA Isolation

Within 48 hours of trapping, rats were stored at either -20°C or -80°C until dissection. We dissected out intact stomachs from each animal and removed the contents. After snap freezing in liquid nitrogen, we homogenised the stomach contents using a sterile mini blender to ensure sampling was representative of the entire stomach.

We purified DNA from 20 mg of homogenised stomach contents using the Promega Wizard Genomic DNA Purification Kit, with the following modifications to the Animal Tissue protocol: after protein precipitation, we transferred the supernatant to a new tube and centrifuged a second time to minimise protein carryover. The DNA pellet was washed twice with ethanol. These modifications were performed to improved DNA

520 purity. We rehydrated precipitated DNA by incubating overnight in molecular biology
521 grade water at 4°C and stored the DNA at -20°C. DNA quantity, purity, and quality was
522 ascertained by nanodrop and agarose gel electrophoresis. The DNA samples were
523 ranked according quantity and purity (based on A260/A280 and secondarily, A230/A280
524 ratios). The eight highest quality DNA samples from each of the three locations were
525 selected for sequencing. We did no size selection on the purified DNA.

526 DNA Sequencing

527 Sequencing was performed on two different dates (24 January 2017 and 17 March
528 2017) using a MinION Mk1B device and R9.4 chemistry. For each sequencing run, DNA
529 from each rat was barcoded using the 1D Native Barcoding Kit (Barcode expansion kit
530 EXP-NBD103 with sequencing kit SQK-LSK108) following the manufacturer's
531 instructions. This included an AMPure bead purification step to remove adaptors, which
532 also likely removed very short reads (less than 200 bp; see **Fig. 1A**). Twelve samples
533 were pooled and run on each flow cell, for a total of 24 individual rats. The flow cells had
534 1373 active pores (January 2017) and 1439 active pores (March 2017). Sequencing
535 was performed using local base calling in MinKnow v1.3.25 (January) or MinKnow
536 v1.5.5 (March), but for the analysis present here both runs were re-basecalled after data
537 collection using Albacore 2.2.7 with demultiplexing performed in Albacore and filtering
538 disabled *(options --barcoding --disable_filtering*).

539 Sequence classification

540 All sequences were BLASTed (blastn v2.6.0+) against a locally compiled database
541 consisting of the combined NCBI other_genomic and nt databases (downloaded on 13th

542 June 2018 from NCBI). Default blastn parameters were used (match 2, mismatch -3,
543 gapopen -5, gapextend -2), and only hits with an e-value of 1e-2 or less were saved.
544 Due to the predominance of short indels present in nanopore sequence data, we used
545 an initial set of basecalled data to test whether changing these default penalties affected
546 the results (gapopen -1, gapextend -1). We found that these adjusted parameters did
547 not qualitatively change our results.

548 We assigned sequence reads to specific taxon levels using MEGAN6 (v.6.11.7 June
549 2018) (41). We only used reads with BLAST hits having an e-value of $1x10^{-20}$ or lower
550 (corresponding to a bit score of 115 or higher given the databases we used) and an
551 alignment length of 100 base pairs or more. To assign reads to taxon levels, we
552 considered all hits having bit scores within 20% of the bit score of the best hit (MEGAN
553 parameter Top Percent).

554 Multivariate analyses

555 Multivariate analyses were done using the software PRIMER v7 (42). The data used in
556 the multivariate analyses were in the form of a sample- (i.e. individual rat) by-family
557 matrix of read counts. All bacteria, rodent, and primate families were removed. The
558 majority of rodent hits were to rat and mouse, resulting from the rats' own DNA (see
559 below). The majority of the primate hits (32 in total) were assigned to Hominidae (19),
560 which likely resulted from sample contamination (**Table S3**).

561 The read counts were converted to proportions per individual rat by dividing by the
562 total count for each rat, to account for the fact that the number of reads varied
563 substantially among rats (43). The proportions were then square-root transformed so

564 that subsequent analyses were informed by the full range of taxa, rather than just the
565 most abundant families (44). We then calculated a matrix of Bray-Curtis dissimilarities,
566 which quantified the difference in the gut DNA of each pair of rats based on the
567 square-root transformed proportions of read counts across families (43).

568 We used unconstrained ordination, non-metric multidimensional scaling (nMDS)
569 applied to the dissimilarity matrix to examine the overall patterns in the diet
570 composition among rats. To assess the degree to which the diet compositions of rats
571 were distinguishable among the three locations, we applied canonical analysis of
572 principal coordinates (CAP) (45) to the dissimilarity matrix. CAP is a constrained
573 ordination which aims to find axes through multivariate data that best separates *a priori*
574 groups of samples (in this case, the groups are the locations from which the rats were
575 sampled); CAP is akin to linear discriminant analysis but it can be used with any
576 resemblance matrix. The out-of-sample classification success was evaluated using a
577 leave-one-out cross-validation procedure (45).

578 We used Similarity Percentage (SIMPER; (46)) to characterise and distinguish between
579 the locations. This allowed us to identify the families with the greatest percentage
580 contributions to (1) the Bray-Curtis similarities of diets within each location (**Table S5**)
581 and (2) the Bray-Curtis dissimilarities between each pair of locations (**Table S6**).

Declarations

Ethics approval

Sample collection was performed under (Auckland Council Permit to Undertake Research WS1064).

Availability of data and materials

Sequence data are available in the SRA archive (accession number PRJEB27647).

Competing interests

WP received funding from Oxford Nanopore Technologies (1000$USD) to present this work at a conference (Ecological Society of Australia 2018).

Funding

This work was supported by a Massey University Research Fund to NF, a Marsden Fund Grant (15-MAU-136) to JD and Marsden Fund Grant (MAU1703) to OS.

Authors' contributions

WP, JD, NF, and OS conceived the project. WP performed the stomach dissections. WP and NF optimised the genomic DNA isolation and library preparation. NF performed the nanopore sequencing. GB and OS processed and performed quality control on the sequencing data. WP and OS performed the sequence classification. WP, AS, NF, and OS analysed the data. WP, NF, AS, and OS wrote the paper, with input from all authors.

Acknowledgements

Thanks to Friends of Okura Bush, Mary Stewart from Auckland Council, and Gillian Wadams and the volunteers at the Waitakere Ranges for collecting rat samples and aiding in rat species identification.

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Supplemental Tables

Table S1. Read numbers and total base pairs for each barcode in the January 2017 sequencing run.

Sample	Total reads	Total Mbp	Mean length
OB2	19907	14.62	734
WP11	10164	9.63	947
WP5	8237	6.78	823
LB7	7548	7.04	933
OB13	3644	3.63	995
WP9	2954	2.4	814
OB5	2850	2.06	721
WP8	2801	2.32	827
LB6	2531	1.6	632
OB7	2473	1.87	756
LB5	1641	1.16	705
LB3	1554	0.99	636
None	16673	13.01	781
Total	82977	67.1	809

788 **Table S2**. Read numbers and total base pairs for each barcode in the March 2017
789 sequencing run.

Sample	Total reads	Total Mbp	Mean length
LB1	17820	9.21	517
LB8	16923	13.13	776
WP2	10511	7.00	666
LB4	8684	4.92	567
OB11	5689	3.40	598
WP10	1563	0.99	633
OB12	1479	0.89	604
WP12	1309	0.78	596
LB2	1127	0.76	676
WP3	637	0.73	1141
OB14	541	0.37	683
OB10	435	0.24	555
None	29432	21.33	725
Total	96150	63.75	663

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Table S3. Characteristics of alignments for reads assigned to the Primate family. Many are both long and have high identity, suggesting that they are not false positive assignments, but contamination.

Rat	Read length	Mean read accuracy	% ID	Alignment length	Genus
WP10	428	0.91	94.0	314	Homo
WP10	782	0.92	93.8	657	Homo
OB5	365	0.90	93.2	249	Homo
LB3	515	0.95	92.6	462	Homo
WP10	510	0.90	92.0	460	Homo
WP10	704	0.90	91.1	587	Homo
WP10	467	0.89	90.3	402	Homo
WP10	494	0.89	89.7	388	Homo
WP10	339	0.88	89.6	269	Homo
WP10	446	0.88	89.6	326	Homo
WP10	327	0.91	89.1	210	Homo
OB5	415	0.89	88.8	277	Homo
OB5	561	0.86	88.7	257	Homo
WP11	434	0.86	88.7	301	Homo
WP10	486	0.88	88.2	365	Homo
WP10	613	0.90	88.2	526	Homo
WP10	563	0.87	88.2	457	Homo
WP10	1373	0.91	87.7	1337	Homo
WP11	526	0.91	87.5	473	Homo
OB14	478	0.89	86.9	373	Homo
OB5	715	0.89	86.8	673	Homo
WP10	475	0.87	86.7	362	Homo
WP10	398	0.86	86.5	259	Homo
WP10	377	0.88	85.3	251	Homo
WP9	558	0.87	85.2	508	Homo
WP10	429	0.86	84.8	276	Homo
WP10	322	0.81	84.5	174	Homo
WP8	723	0.84	83.1	438	Homo
LB8	965	0.86	80.4	245	Rhinopithecus

LB5	3042	0.94	79.4	1018	Cebus
WP2	464	0.93	77.3	216	Homo
LB8	671	0.90	73.9	406	Aotus

797 **Table S4. Characteristics of alignments for reads that are likely false positive**
798 **assignments.** Most are short long or have low identity, suggesting that they are false
799 positive assignments. The exception are the reads matching Buthidae, which we
800 hypothesize are due to the rats predation of the sister taxa, harvestmen.

Rat	Read length	Mean read accuracy	% ID	Alignment length	Genus	Megan family
WP5	1285	0.90	82.9	298	Centruroides	Buthidae
OB11	1874	0.90	88.0	664	Centruroides	Buthidae
WP5	1711	0.93	79.3	762	Centruroides	Buthidae
WP11	859	0.93	86.1	151	Octopus	Octopodidae
WP2	516	0.86	81.4	172	Oncorhynchus	Salmonidae
OB12	424	0.90	85.7	140	Xiphophorus	Poeciliidae
OB12	643	0.84	89.3	177	Xiphophorus	Poeciliidae

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802 **Table S5.** SIMPER analysis of family contributions to group similarities.

Family	Average	Average	Similarity/SD	Percentage	Group
Hymenolepididae	3.37	6.87	0.34	51.2	LB
Solanaceae	1.57	1.48	0.34	11.1	LB
Fabaceae	1.74	1.41	0.44	10.5	LB
Arecaceae	2.86	7.11	1	33.4	OB
Poaceae	2.87	4.82	0.55	22.7	OB
Fabaceae	1.17	1.98	0.51	9.3	OB
Phasianidae	1.79	1.67	0.34	7.9	OB
Poaceae	5.08	17.61	0.62	72.1	WP

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Table S6. SIMPER analysis of family contributions to group dissimilarities.

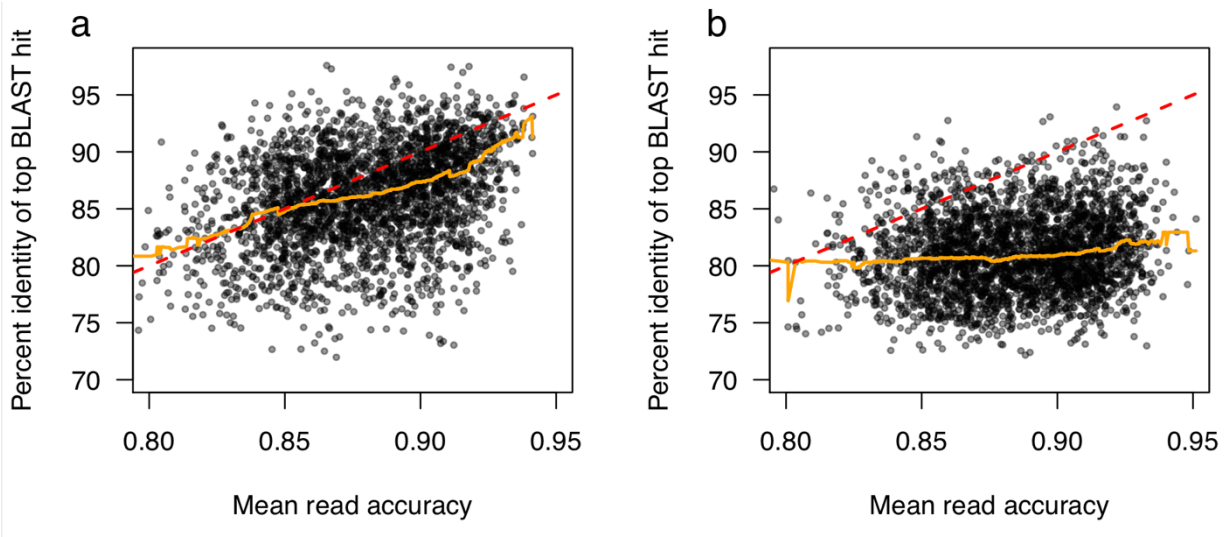
Species	Av Abund Group1	Avg Abund Group2	Avg. Diss	Diss/SD	% contrib	Group 1	Group2
Poaceae	1.95	5.08	15.15	1.04	16.74	LB	WP
Poaceae	2.87	5.08	11.29	1.26	13.78	OB	WP
Hymenolepididae	3.37	0.48	10.8	0.73	11.93	LB	WP
Hymenolepididae	3.37	0.29	9.37	0.79	10.32	LB	OB
Poaceae	1.95	2.87	8.37	1.1	9.22	LB	OB
Arecaceae	0.05	2.86	6.99	1.41	7.7	LB	OB
Arecaceae	2.86	1.31	5.92	1.29	7.23	OB	WP
Fabaceae	1.74	1.05	6.14	0.67	6.78	LB	WP
Podocarpaceae	0	2.38	5.34	0.83	5.9	LB	WP
Podocarpaceae	0.71	2.38	4.82	0.99	5.88	OB	WP
Fabaceae	1.74	1.17	4.87	0.81	5.37	LB	OB
Fabaceae	1.17	1.05	4.31	0.84	5.26	OB	WP

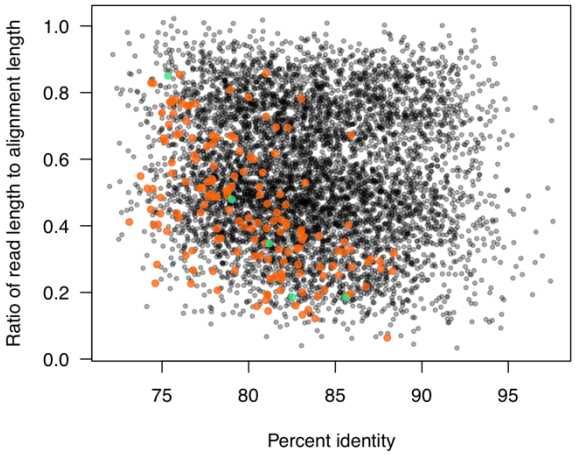
Datafile S1. Table of read BLAST hits and assigned MEGAN taxa with with reads reclassified at the family or order level by filtering on read length to alignment length ratio and percent identity.

Datafile S2. Table of read BLAST hits and assigned MEGAN taxa with no filters applied.

Supplemental Figures

Fig S1. Correlation of read accuracy with alignment characteristics. Only rat reads exhibit a clear positive relationship between accuracy and percent identity. (A) indicates the relationship for reads assigned to *Rattus* and (B) for *Mus*. The orange line indicates a running median; the red dotted line is the y=x line, which is expected if accuracy corresponds exactly to percent identity.



822 **Fig S2. Alignment characteristics of true positive and false positive taxon**
823 **assignments at the family level**. False positive taxon assignments (*Cricetideaa*,
824 orange, and *Spalacidae*, green) have lower percent identity and shorter alignment
825 lengths than true positive taxon assignments (*Muridae*, black). Only a single false
826 positive taxon assignment has a read length to alignment length ratio greater than 0.5
827 and a percent identity greater than 85%. This suggests that with further filters or
828 methodologies (e.g. decision tree analysis using different read and alignment
829 characteristics) could, if necessary, decrease false positive rates even further.

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